REMARKS

Status of the claims

Claims 1, 6, 21, 22, 25-37 are pending.

By entry of this amendment, claims 21-22 have been canceled without disclaimer or prejudice. Applicants reserve the right to pursue the subject matter encompassed in the canceled claims in subsequent continuation or divisional applications.

Claims 1, 28, and 33 have been amended by this amendment. Claim 6 and 22 is as originally filed. Thus, claims 1, 6, 25-37 are currently under examination.

No new matter has been added by these amendments. Support for amended claims can be found in the example section, Table 2, Table 5, Table 7, and the Sequence Listing.

This amendment adds, changes and/or deletes claims in the instant application. A detailed listing of all claims that are, or were, in the application, irrespective of whether the claims remain under examination in the application, are presented with an appropriate defined status identifiers. See 37 C.F.R. §1.121(c).

Withdrawn Objections and Rejections

Applicants would like to thank the Examiner for withdrawing the objections and rejections as stated in the Final Office Action dated September 10, 2007.

Rejection under 35 USC §112, first paragraph, enablement

The claims stand rejected under 35 USC §112, first paragraph, for allegedly being not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention. Applicants respectfully traverse.

The Examiner stated that one of the two samples in the Example section of the specification did not show significance. There is no dispute, however, that the other sample, S0012, shows that the claimed SNP is significantly associated with coronary stenosis. See Table 7.

Applicants hereby submit additional studies published earlier this year showing that the instantly claimed SNP, called LPA I4399M (rs3798220) in the published article, has been associated with Severe Coronary Artery Diseases in three different samples. See Table 2 and Table 3 of the

attached article by Luke, et al., Arteriosclerosis, Thrombosis, and Vascular Biology, 2007;27:2030-2036).

The studies conducted in the published article genotyped the patients individually, as opposed to the pool studies as shown in the Example sections. Thus, it is conceivable that the p-values might be different, even if the sample sets they were derived from are the same.

In addition, the claims have been amended adding the language encompassing the complementary strand of SEQ ID NO: 19350. Thus, the claim language directed towards the presence of A allele being associated with decreased risk is well supported by Table 7, which shows T, the allele on the complementary strand of the A allele, is indeed associated with decreased risk (odds ratio less than 1).

Therefore, the rejection under 35 USC §112, first paragraph, for allegedly lack of enablement has been overcome. The Examiner is respectfully requested to withdraw the rejection.

Rejection under 35 USC §112, first paragraph, new matter

The claims stand rejected under 35 USC §112, first paragraph, for allegedly containing new matter. Applicants respectfully traverse.

The Examiner asserted that the specification did not support the language encompassing the presence of G at position 101 of SEQ ID NP: 19350 being associated with increased risk of coronary stenosis.

As disclosed in the primer table, Table 5 of the specification, the two alleles of the instant claimed SNP are C and T. Table 7 shows that the T allele is associated with decreased risk, thus making the C allele being associated with increased risk, as the risk association in the case-control study is relative to each other. Therefore, the allele on the complementary strand of the C allele would be G.

Thus, the rejection under 35 USC §112, first paragraph, for allegedly containing new matter has been overcome. The Examiner is respectfully requested to withdraw the rejection.

Rejection under 35 USC §103

Claims 21 and 22 stand rejected under 35 USC §103, for allegedly being obvious over SNP rs3798220 in view of Nollau et al.

Claims 21 and 22 have been canceled, thus making the rejection moot. The Examiner is respectfully requested to withdraw the rejection.

In conclusion, in light of the amendments and remarks above, Applicants submit that the present application is fully in condition for allowance. Early notice to that effect is earnestly requested.

The Examiner is invited to contact the undersigned via telephone if a phone interview would expedite the prosecution of the instant patent application.

Respectfully submitted,

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Enclosure : Luke, et al., Arterioscler. Thromb. Vasc. Biol. 2007 ;27 :2030-2036.

Arteriosclerosis, Thrombosis, and Vascular Biology

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A Polymorphism in the Protease-Like Domain of Apolipoprotein(a) Is Associated With Severe Coronary Artery Disease

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Arterioscler. Thromb. Vasc. Biol. 2007;27;2030-2036; originally published online Jun 14, 2007;
DOI: 10.1161/ATVBAHA.107.141291
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association. 7272 Greenville Avenue, Dallas, TX 72514
 Print ISSN: 1079-5642. Online ISSN: 1524-4636

A Polymorphism in the Protease-Like Domain of Apolipoprotein(a) Is Associated With Severe Coronary Artery Disease

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Objectives—The purpose of this study was to identify genetic variants associated with severe coronary artery disease (CAD).

Methods and Results—We used 3 case-control studies of white subjects whose severity of CAD was assessed by angiography. The first 2 studies were used to generate hypotheses that were then tested in the third study. We tested 12 077 putative functional single nucleotide polymorphisms (SNPs) in Study 1 (781 cases, 603 controls) and identified 302 SNPs nominally associated with severe CAD. Testing these 302 SNPs in Study 2 (471 cases, 298 controls), we found 5 (in *LPA*, *CALM1*, *IIAP1*, *AP3B1*, and *ABCG2*) were nominally associated with severe CAD and had the same risk alleles in both studies. We then tested these 5 SNPs in Study 3 (554 cases, 373 controls). We found 1 SNP that was associated with severe CAD: *LPA* I4399M (rs3798220). *LPA* encodes apolipoprotein(a), a component of lipoprotein(a). I4399M is located in the protease-like domain of apolipoprotein(a). Compared with noncarriers, carriers of the 4399M risk allele (2.7% of controls) had an adjusted odds ratio for severe CAD of 3.14 (confidence interval 1.51 to 6.56), and had 5-fold higher median plasma lipoprotein(a) levels (P=0.003)

Conclusions—The *LPA* I4399M SNP is associated with severe CAD and plasma lipoprotein(a) levels. (***Arterioscler Thromb Vasc Biol.* 2007;27:2030-2036.**)

Key Words: coronary arteriosclerosis ■ genetics ■ single nucleotide polymorphism ■ lipoprotein(a) ■ risk factors

Severe coronary artery disease (CAD), characterized by occlusive epicardial coronary stenosis, and its consequences such as myocardial infarction (MI) are the leading causes of death in the United States.[1] Several major risk factors for coronary disease are well established and form the basis of current risk assessment algorithms.[2,3] However, some risk factors for coronary disease have not yet been identified, because some of the patients with coronary disease do not have traditional risk factors,[4] and traditional risk factors do not reliably predict premature MI.[5] The unidentified risk factors probably include genetic variants because genetics is considered to have an important role in coronary disease,[6,7] and a family history of cardiovascular disease is an independent risk factor.[8] One approach to identify genetic variants associated with complex diseases, such as coronary disease, is to use multiple association studies. We have previously identified genetic variants associated with MI and early-onset MI by testing thousands of putative functional SNPs in 3 case-control studies.[7,9] Thus, we have taken the same approach for angiographically defined severe CAD in 3 case-control studies, and asked if we could identify genetic variants associated with severe CAD.

Methods

Study Design

Because testing 12 077 SNPs for association with severe CAD could result in false-positives, we used 3 consecutive case-control studies. We generated a limited number of hypotheses in the first 2 studies by identifying a subset of SNPs that were nominally associated with severe CAD and had the same risk alleles in both studies and then tested these hypotheses in a third study.

Angiographic Assessment of CAD Severity

The severity of CAD was assessed by scoring the angiograms of subjects who had undergone clinically indicated coronary angiogra-

Original received February 5, 2007; final version accepted May 23, 2007.

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Arterioscler Thromb Vasc Biol. is available at http://atvb.ahajournals.org DOI: 10.1161/ATVBAHA.107.141291

TABLE 1. Clinical Characteristics of Cases and Controls in Study 1, Study 2, and Study 3

	Study 1			Study 2			Study 3		
Characteristic	Cases (n=781)	Controls (n=603)	*P* Value	Cases (n=471)	Controls (n=298)	*P* Value	Cases (n=554)	Controls (n=373)	*P* Value
Stenosis score*	270 (200–350)	0	N/A	355 (303–434)	0 (0–35)	N/A	300 (250–375)	0	N/A
Age, years	60±8	59±11	N/A	61±11	58±12	N/A	63±8	61±9	N/A
Male sex	480 (61)	376 (62)	N/A	252 (54)	166 (56)	N/A	358 (65)	164 (44)	N/A
Smoking†	531 (68)	326 (54)	<0.001	300 (64)	156 (52)	0.002	365 (66)	189 (51)	<0.001
Diabetes‡	286 (37)	63 (10)	<0.001	100 (21)	19 (6)	<0.001	230 (42)	54 (14)	<0.001
Hypertension‡	735 (94)	469 (78)	<0.001	297 (63)	135 (45)	<0.001	524 (95)	310 (83)	<0.001
Dyslipidemia‡	733 (95)	344 (59)	<0.001	411 (87)	183 (62)	<0.001	512 (94)	253 (70)	<0.001
BMI, kg/m²	31±6	30±7	<0.001	28±5	27±5	0.06	30±6	30±7	0.48

Data presented as median (interquartile range) for stenosis score, mean±standard deviation for Age and BMI, or No. (%) of subjects for the other characteristics. N/A indicates that *P* values were not calculated because the characteristic was considered during the selection of cases and controls. *P* values are from Fisher exact test, except those for BMI, which are from the Wilcoxon rank sum test. BMI indicates body mass index.

*Calculation of the stenosis score is presented in supplemental Methods.

†Current or past smoking.

‡Subjects were considered to have this risk factor if the questionnaire indicated medical treatment for or a history of this risk factor.

phy. The severity of CAD was defined by a stenosis score calculated as the sum of the maximum percent stenosis in 10 coronary artery segments: the left main and 3 segments (proximal, medial, distal), each of the left anterior descending, left circumflex, and right coronary arteries. Details of the angiographic assessment of CAD and scoring methods used in these studies are described in the supplemental Methods (available online at http://atvb.ahajournals.org).

Study Subjects

Subjects in all 3 studies were unrelated women and men who had undergone coronary angiography (characteristics of cases and controls are presented in Table 1). Three goals of our study design influenced the choice of the stenosis score limits and the age limits used to select cases and controls. The first goal was to compare cases and controls at the extreme ends of the stenosis phenotype; the second goal was to include a large number of subjects; and the third goal was to select case and control groups that were about 40% or more female. Because males generally have higher stenosis score than females and have severe CAD at younger ages than females, we set stenosis score limits and age limits separately for males and females. Details of inclusion and exclusion criteria as well as stenosis score limits and age limits are described in supplemental Methods.

Subjects in Study 1 and Study 3 were drawn from the Cleveland Clinic Foundation (CCF) Genebank and included only those who selected Eastern European, Northern European, or "Caucasian Other" as the ethnicity for both parents. Study 1 comprised 781 cases and 603 controls selected from angiography patients enrolled in the CCF Genebank between December 2000 and March 2003 and whose DNA samples arrived at Celera before October 2003. Study 3 comprised 554 cases and 373 controls enrolled in the CCF Genebank between July 2001 and December 2003 and whose DNA samples arrived at Celera after August 2004. Subjects in Study 2 were drawn from Genomic Resource at University of California San Francisco (UCSF) and included those who selected only white as their ethnicity. Study 2 comprised 471 cases and 298 controls drawn from angiography patients enrolled between June 1990 and March 2003.

An additional group of 485 subjects who were not in Study 1, Study 2, or Study 3 were used to investigate the association between genotype and Lp(a) levels. These subjects had Lp(a) levels available in the database of the UCSF Genomic Resource and were drawn from the subjects of a previously published genetic study of MI.[9] The clinical characteristics of these 485 subjects are presented in supplemental Table 1. Most of the Study 1 subjects (444 cases with a history of MI and 602 controls) and more than half (486 of 769) of Study 2 subjects, but none of the Study 3 subjects, were also subjects in the previously published genetic study of MI.[9]

All subjects gave informed consent and completed an Institutional Review Board approved questionnaire.

SNPs Tested

We tested 12 077 SNPs in Study 1. These putative functional SNPs are in 7439 genes, and 70% of the SNPs modify the amino acid sequence of the encoded proteins; the rest are potential regulatory SNPs (3′ or 5′ untranslated regions, transcription factor binding sites, or exon splice sites). Additional SNPs in the LPA gene were selected using Tagger[10] as implemented in Haploview.[11]

Genotyping and Laboratory Measurements

Genotypes for individual DNA samples were determined by real-time kinetic polymerase chain reaction (PCR) as described previously.[9] Allele frequencies of SNPs were determined in Study 1 and Study 2 using pooled DNA samples as previously described.[9] The plasma Lp(a) levels in units of nmol/L were determined by an ELISA method as previously described.[12] The size of apo(a) isoforms, reported as the number of KIV repeats in apo(a), was determined by immunoblotting as previously described.[13] Further details of these methods are described in supplemental Methods.

Statistical Analysis

Subject characteristics were summarized by disease status for each study, and differences were assessed using Fisher exact test or the Wilcoxon rank sum test for discrete and continuous characteristics, respectively. A chi-square test was used to assess allele frequency differences that were based on data from pooled DNA samples, and Fisher exact test was used to assess allele frequency differences that were based on genotyping results. An exact test was used to assess deviation of genotype frequencies from Hardy-Weinberg expectations.[14] When logistic regression was used to estimate odds ratios, significance was assessed using the Wald test. When risk alleles for severe CAD were prespecified based on Study 1 results for SNPs, the association of risk alleles with severe CAD was assessed in subsequent studies using 1-sided probability values and 90% confidence intervals (because there was 95% confidence that the true risk estimates were greater than the lower bounds of the 90% confidence intervals). All other probability values are 2-sided and 95% confidence intervals are presented. Likelihood ratio tests were used to evaluate potential interactions between genotype and each traditional risk factor in separate regression models that included an interaction term between genotype and the covariate of interest. The association

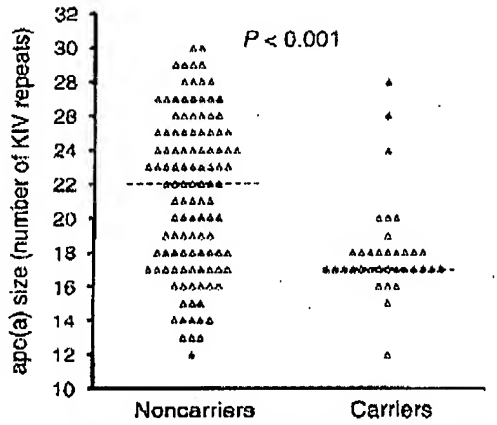

Figure 1. Association of the *LPA* I4399M SNP with apo(a) isoform size. Plasma apo(a) isoform sizes were determined for 114 noncarriers and 35 carriers of *LPA* 4399M in Study 2. Carriers of the 4399M risk allele had significantly smaller apo(a) isoforms. Individual apo(a) isoform sizes (indicated by Δ) are reported as the number of KIV repeats in the apo(a) isoform, and the median sizes are indicated by the dashed lines.

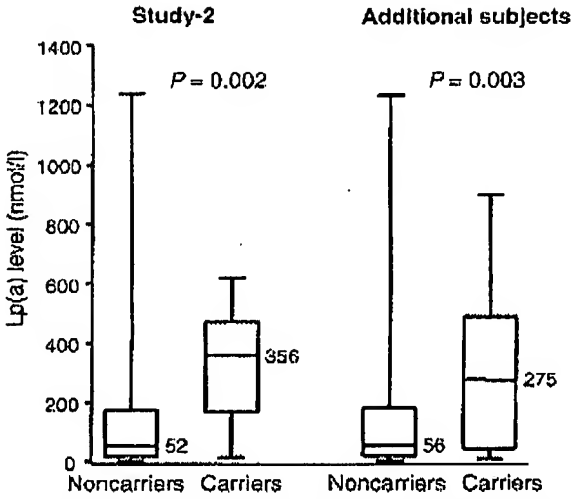

Figure 2. Association of the *LPA* I4399M SNP with plasma Lp(a) levels. In 161 Study 2 subjects for whom plasma Lp(a) levels were available, carriers of the *LPA* 4399M allele (n=12) had higher Lp(a) levels than did noncarriers (n=149). In an additional 485 subjects for whom plasma Lp(a) levels were available, carriers of the *LPA* 4399M allele (n=21) also had higher Lp(a) levels than did noncarriers (n=464). The median values are shown next to the boxes and indicated by the horizontal lines inside the boxes. The boxes extend from the 25th to 75th percentile and the whiskers extend from the lowest to the highest value.

of LPA I4399M genotype with apo(a) isoform size (Figure 1) and untransformed Lp(a) plasma levels (Figure 2) were assessed with the Wilcoxon rank sum test. A multiple linear regression model was used to estimate the relationship between the *LPA* I4399M carrier status and the ln of Lp(a) plasma levels while adjusting for the effect of apo(a) isoform size. The ln transformed Lp(a) levels were used in the linear regression analysis so that the distribution of the residuals more closely approximated a Gaussian distribution.

Results

LPA I4399M Is Associated With Severe CAD

The demographic and clinical characteristics of the subjects of Study 1, Study 2 and Study 3 are summarized in Table 1.

We measured the allele frequencies of 12 077 putative functional SNPs in Study 1 cases and controls using pooled DNA samples and identified 302 SNPs that were nominally associated with severe CAD ($P<0.05$) and had odds ratios for severe CAD of greater than 1.3 and had minor allele frequency estimates that were greater than 2% (supplemental Table II). For these 302 SNPs, we determined allele frequencies in Study 2 cases and controls using pooled DNA samples and asked if the risk allele identified in Study 1 was also associated with severe CAD in Study 2. For SNPs that were associated with severe CAD and had the same risk alleles in both pooling studies, we then confirmed their allele frequencies by genotyping individual DNA samples from Study 1 and Study 2 subjects. We found that the risk alleles of 5 SNPs in 5 genes were nominally associated ($P<0.05$) with severe CAD in both studies (Table 2). The genes encoded apolipoprotein(a) (encoded by *LPA*), calmodulin 1 (*CALM1*), huntingtin-associated protein 1 (*HAP1*), adaptor-related protein complex 3, β-1 subunit (*AP3B1*), and ATP-binding cassette, subfamily G, member 2 (*ABCG2*). The genotype distributions of these 5 SNPs in the control groups of Study 1 and Study 2 did not deviate from Hardy-Weinberg equilibrium expectations ($P>0.05$).

After prespecifying the risk alleles based on Study 1 and Study 2 results, we tested the hypotheses that the risk alleles of these 5 SNPs would be associated with severe CAD in Study 3. We found that the risk allele of 1 of the 5 SNPs, I4399M (rs3798220) in the *LPA* gene, was associated ($P<0.05$) with severe CAD. The *LPA* gene encodes apolipoprotein(a) (apo(a)), which is a component of lipoprotein(a) (Lp(a)), and the I4399M SNP is located in the protease-like domain of apo(a). Carriers of the 4399M allele constituted 2.7% of controls and 5.2% of cases in Study 3. Compared with noncarriers, carriers of the 4399M risk allele had an odds ratio for severe CAD of 3.14 (CI 1.51 to 6.56, $P=0.005$, Table 3) after adjusting for traditional risk factors (age, sex, smoking, hypertension, diabetes, dyslipidemia, and body mass index [BMI]). This association remained significant ($P=0.026$) after Bonferroni[15] correction for testing 5 SNPs in Study 3. We observed no indication of an interaction between the I4399M genotype and age, sex, smoking, diabetes, dyslipidemia, or BMI in Study 3 ($P>0.11$), but we did observe an interaction between genotype and hypertension ($P=0.02$). However, when we tested for interaction between I4399M genotype and hypertension in Study 1 and Study 2 we did not observe significant interactions ($P=0.94$ and $P=0.78$, respectively).

Genetic Variants in Linkage Disequilibrium With *LPA* I4399M

We used 2 approaches to investigate whether the association of *LPA* I4399M with severe CAD could be due to linkage disequilibrium (LD) between I4399M and other variants in the *LPA* gene. In the first approach, we asked whether other SNPs in the *LPA* gene were associated with severe CAD and

TABLE 2. Unadjusted Association of 5 SNPs With Severe CAD in Study 1 and Study 2

SNP ID	Gene Symbol	Chromosome	Study	Major Allele*	Minor Allele*	Type of SNP*	Case AF†	Control AF†	OR‡	CI	P Value§
rs3798220	*LPA*	6	1	A	G	I4399M	0.04	0.01	3.79	1.97–7.29	<0.001
			2	A	G		0.04	0.02	2.25	1.27–3.97	0.010
rs3814843	*CALM1*	14	1	T	G	3′UTR	0.05	0.03	1.66	1.11–2.40	0.012
			2	T	G		0.06	0.04	1.74	1.13–2.67	0.020
rs4796603	*HAP1*	17	1	A	T	T58S	0.83	0.79	1.34	1.10–1.63	0.004
			2	A	T		0.83	0.78	1.36	1.09–1.68	0.012
rs6453373	*AP3B1*	5	1	A	T	E585V	0.94	0.92	1.51	1.11–2.04	0.008
			2	A	T		0.93	0.90	1.50	1.09–2.05	0.022
rs2231137	*ABCG2*	4	1	G	A	V12M	0.97	0.95	1.60	1.08–2.37	0.020
			2	G	A		0.96	0.94	1.62	1.10–2.38	0.028

*The polymorphic nucleotides on the sense strands are shown. Major alleles are on the left, and the risk alleles are bolded.
†Allele frequency for the risk allele.
‡Allelic odds ratios for the risk allele.
§For Study 1, 2-sided *P* values and 95% confidence intervals are reported. For Study 2, where the risk alleles have been prespecified based on Study 1, 1-sided *P* values and 90% confidence intervals are reported.
3′UTR indicates 3′ untranslated region.

could explain the association of I4399M with severe CAD. The HapMap project reports 65 SNPs in the *LPA* gene that have allele frequencies >2% in the CEU population (Utah residents with ancestry from northern and western Europe, HapMap public release #21[16]). We identified a set of 18 SNPs that tagged 50 of these 65 SNPs with an r^2 >0.80, 12 SNPs with an r^2 <0.8 but >0.5, and 3 SNPs with an r^2 <0.5. We then genotyped the subjects of Study 1 (the largest of the 3 studies) for these 18 SNPs and the I4399M SNP which tags only itself. Except for the I4399M SNP, none of these 18 additional tagging SNPs was associated with severe CAD after adjusting for traditional risk factors (supplemental Table III). In Study 1 the I4399M SNP is not in strong LD with any of the other 18 tagging SNPs (r^2 ≤0.1), and the HapMap project does not report LD for the LPA I4399M SNP because that position is not polymorphic in the 30 CEU trios (60 parents and 30 offspring) genotyped by the HapMap project.

We also investigated whether the association of the *LPA* I4399M SNP with severe CAD could be attributable to LD between I4399M and the repeat polymorphism in the LPA gene that encodes the kringle IV (KIV) repeat length variation. This variation determines apo(a) isoform size which has been previously shown to be associated with coronary disease.[17] Direct determination of KIV repeat length in the *LPA* gene requires nucleated cells which were not available for these studies.[18] However, the KIV repeat length can also be determined from the number of KIV repeats in the apo(a) isoforms present in stored plasma.[19] Because stored plasma was available for some of the Study 2 subjects, we calculated the number of subjects needed to have 80% power to detect an association between the I4399M SNP and apo(a) isoform size (supplemental Methods). We then determined apo(a) isoform size for 35 carriers and 114 noncarriers of 4399M among Study 2 subjects. We found that in this group of 149 subjects, the I4399M SNP genotype was associated with apo(a) isoform size: the median apo(a) isoform size in carriers contained 17 KIV repeats and in noncarriers, 22 KIV repeats (P<0.001, Figure 1). However, in this group of 149 subjects, the association of the *LPA* 4399M allele with severe CAD remained significant after adjusting for the apo(a) size (odds ratio=4.36, CI 1.53 to 12.4, P=0.006; supplemental Table IV). Thus, we found no evidence that the association between the *LPA* 4399M allele with CAD is explained by apo(a) size polymorphism.

Plausibility of the Association of *LPA* I4399M With Severe CAD

To investigate the biological plausibility of the association between the *LPA* I4399M SNP and severe CAD, we asked

TABLE 3. Association of *LPA* I4399M With Severe CAD in Study 3

			Unadjusted			Adjusted§		
Genotype	Case*	Control*	OR†	CI‡	P Value‡	OR†	CI‡	P Value‡
MM	1 (0.2)	0 (0.0)	...	...	...	...	...	...
IM	28 (5.1)	10 (2.7)	1.94	1.05–3.59	0.039	3.09	1.48–6.48	0.006
MM+IM	29 (5.2)	10 (2.7)	2.01	1.09–3.70	0.031	3.14	1.51–6.56	0.005
II	525 (94.8)	363 (97.3)	1.00	reference		1.00	Reference	

*Data presented as No. (%) of subjects.
†Odds ratios were estimated by logistic regression.
‡*P* values (Wald test) are 1-sided and 90% CI are presented because the risk allele was prespecified.
§Adjusted for age, sex, smoking, diabetes, dyslipidemia, hypertension, and BMI.

whether the SNP was associated with plasma levels of Lp(a), which have been associated with coronary disease.[20] Plasma Lp(a) levels were available in the UCSF Genomic Resource database for 161 subjects of Study 2 (these 161 subjects included 122 of the subjects shown in Figure 1; plasma Lp(a) levels were not available for Study 1 or Study 3 subjects). In these 161 subjects of Study 2, we found that Lp(a) levels were higher in carriers of the 4399M allele than in noncarriers (P=0.002): median levels were 356 nmol/L and 52 nmol/L, respectively (Figure 2). To confirm this result, we tested the association of the I4399M SNP with Lp(a) levels in 485 additional subjects with available Lp(a) levels (characteristics of these subjects are presented in supplemental Table I). These 485 subjects had not been included in Study 1, Study 2, or Study 3. In these 485 additional subjects, we again found that the Lp(a) levels were higher in carriers of the 4399M allele than in noncarriers (P=0.003, Figure 2).

We also asked whether the association of I4399M with Lp(a) levels can be explained by the association of I4399M with apo(a) size. Of the 161 Study 2 subjects who had Lp(a) levels available (left panel of Figure 2), 122 also had apo(a) size information available from the analysis in Figure 1. In these 122 subjects, we found that Lp(a) levels were 5.9-fold higher in carriers of the 4399M allele than in noncarriers, corresponding to a 1.78-ln unit increase in Lp(a) levels (P=0.002; supplemental Table V), and after adjusting for apo(a) size, Lp(a) levels remained 3.7-fold higher in carriers than in noncarriers, corresponding to a 1.32-ln unit increase in Lp(a) levels (P=0.013; supplemental Table V).

Discussion

We found that a genetic variant of *LPA*, the I4399M SNP, is associated with severe CAD. Carriers of the 4399M risk allele constituted 2.7% of the control subjects and had an adjusted odds ratio for severe CAD of 3.14 (90% CI 1.51 to 6.56; Table 3). This association seems unlikely to be a false-positive finding because it remained significant after correcting for multiple testing.

The *LPA* gene encodes the apo(a) protein of the Lp(a) particle, and high plasma Lp(a) levels are considered an emerging lipid risk factor for cardiovascular disease.[3,21] The variability in plasma Lp(a) levels among individuals are largely determined by genetic variations at the *LPA* gene locus,[22] a fraction of that variability has been attributed to variation in apo(a) size[22,23] resulting from the KIV type-2 repeat polymorphism.[19] The apo(a) protein in apparently healthy European Caucasians has been previously reported to contain a median of 27 KIV repeats.[24] The somewhat lower number of KIV repeats we observed in noncarriers (22 repeats) may reflect the higher than normal risk status of the subjects of our studies; all underwent clinically indicated coronary angiography. A number of other polymorphisms in the kringle region and in the 5′ noncoding region have also been reported to be associated with Lp(a) levels.[20,25–30]

We did not find evidence that the association of *LPA* I4399M with severe CAD was attributable to other variants in the *LPA* gene. We investigated 18 additional SNPs in the *LPA* gene that tagged 50 of the 65 SNPs that have allele frequency >2% in the HapMap CEU population. These 18 SNPs included 2 SNPs, T3907P and L3866V (same as T3888P and L3847V in Chretien et al), which have recently been reported to be associated with Lp(a) levels.[30] We found that none of these 18 SNPs could explain the association of LPA I4399M SNP with severe CAD. We also found that the apo(a) isoform size did not explain the association of *LPA* I4399M SNP with severe CAD.

Although we tested 12 077 putative functional SNPs from more than 7000 genes, the one genetic variant that remained associated with severe CAD in all 3 studies was the I4399M SNP in *LPA*, a gene that has often been implicated in vascular disease.[21] Thus, the association of *LPA* I4399M with severe CAD is biologically plausible both because *LPA* is a candidate gene for cardiovascular disease and also because this SNP is associated with Lp(a) levels (Figure 2). Whether or how the isoleucine to methionine substitution directly affects Lp(a) levels or CAD risk is not known. It is interesting to note that in apolipoprotein A-I, the oxidation of methionine residues has been shown to alter the sites and rates of the proteolytic cleavage of apolipoprotein A-I.[31] Thus we could speculate that potential oxidation of the 4399 methionine residue could alter apo(a) and Lp(a) catabolism, eg, by altering proteolytic fragmentation of either free or LDL-bound apo(a),[32] hence altering Lp(a) levels. Alternatively, it has been suggested that Lp(a) plays a role in fibrinolysis[21] and that it may be a carrier for proinflammatory and oxidized phospholipids[33]; both of these roles could conceivably be affected by a methionine substitution and its potential oxidation in the protease-like domain of apo(a). It would therefore be interesting to investigate the potential role of the I4399M SNP in Lp(a) physiology either in vitro or in transgenic animal models that overexpress the 2 I4399M alleles. Nevertheless, given that determining the KIV repeat length in the *LPA* gene or the apo(a) size in plasma requires more specialized techniques and samples that may not be available, the association of I4399M with apo(a) size could provide an alternative approach for obtaining information related to KIV repeat length or apo(a) size.

Results in this report contain several attributes that are considered desirable for a genetic association study,[34] including biological rationale, rigorous phenotyping and genotyping, multiple large sample sets, correction of probability values for multiple testing, and physiologically meaningful supporting evidence. It is worth noting that the I4399M SNP, which we found to be associated with severe CAD as well as with Lp(a) levels, has a relatively low frequency of about 2% in the control group. This finding suggests the need for designing sequencing projects with adequate power to detect SNPs of similar frequency. However, possible limitations include the inability of coronary angiography to identify circumferential disease; thus the stenosis score may have underestimated the extent of CAD for some of the control subjects. In addition, in Study 2 we tested only those SNPs that had had an odds ratio for severe CAD of greater than 1.3 in Study 1. Furthermore, even for SNPs with a true OR of 1.3, we had 80% power to detect association with severe CAD in Study 1 only for SNPs with minor allele frequencies of 0.2 or higher. This combination of a power limitation for low frequency SNPs in Study 1 and the odds-ratio cutoff we used

to advance SNPs from Study 1 to Study 2 could have lead to false-negative results. Our analyses of Lp(a) levels and apo(a) size were restricted to those limited to a subset of subjects that had Lp(a) levels in the database and our analyses of apo(a) sizes were restricted to a subset of those subjects for whom plasma samples were in storage, and not all of these subjects had Lp(a) levels available. Apo(a) size determined from stored plasma may not fully reflect the genetic variability of the KIV repeat length polymorphism because larger apo(a) isoforms are secreted into the plasma at lower levels.[35] However, we could not directly determine the KIV repeat length in the *LPA* gene because nucleated cells were required but were not available. Finally, these results were derived from case-control studies of white subjects; thus the association of the *LPA* I4399M SNP with severe CAD and Lp(a) levels should be investigated in other ethnic groups and in prospective population-based cohorts.

In conclusion, we found that the I4399M genetic variant of *LPA* is associated with severe CAD, and the association remained significant after adjusting for multiple testing. The plausibility of this association is supported by the association of I4399M with Lp(a) levels. Functional studies of the *LPA* 4399M variant could shed light on the role of Lp(a) in the pathophysiology of vascular disease.

Acknowledgments

The authors thank Thomas White, John Sninsky, Lance Bare, Olga Iakoubova, and Bradford Young for helpful comments, and Judy Louie, David Ross, Alla Smolgovsky, Joel Bolonick, and Steve Schrodi for data and statistical analyses. The authors are grateful to the subjects of the genetic association studies.

Sources of Funding

This study received funding from the University of California Discovery Grant Program, which is jointly funded by the University of California and the State of California with matching funds from Celera.

Disclosures

M.L., D.M.L., C.R., D.S., J.C., D.U.L., A.A., C.T., and J.D. are current or former employees of Celera. J.K. and M.M. received funding from the University of California Discovery Grant Program which is jointly funded by the University of California and the State of California with matching funds from Celera. S.E. had been a paid consultant of Celera.

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